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Chromogenic Endotoxin Assay in Plasma
Selection of Plasma Pretreatment and Production of Standard Curves

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Summary: The aim of this study was to define the optimal conditions for the plasma pretreatment and to improve the production of standard curves for plasma endotoxin determination by a chromogenic substrate assay. Endotoxin standard from *E. coli* O 111 : B 4 (0–50 ng/l) was added to pyrogen-free water or to plasma samples from 12 healthy subjects and 24 alcoholics, before pretreatment by heating (75 °C, 5 minutes) or with perchloric acid (0.32 mol/l). When endotoxin standard curves were determined using a microprocessor-controlled reader, the slopes of the curves obtained with plasma differed from those with pyrogen-free water. The slope of the standard curve prepared with plasma samples from different patients exhibited marked interindividual variations. Compared with the heating method, the perchloric acid method gave more variable results and a lower recovery of added endotoxin, especially in plasma from alcoholics. The results permit the following conclusion:

1. For plasma endotoxin determination, a standard curve should be prepared for each individual plasma sample.

2. The endotoxin standard should be added before pretreatment of the plasma.

3. Pretreatment of the plasma by heating at 75 °C for 5 minutes provides more reliable results than pretreatment with perchloric acid.

Introduction

Numerous studies on the determination of endotoxin in human plasma have been published (1, 2). Most have been performed using different variations of the classical *Limulus* lysate test (measurement of gel formation). The problems associated with this procedure have been discussed in detail elsewhere (3). For quantitative measurements of endotoxin in plasma, a chromogenic method has been described by Japanese authors (4). Although the chromogenic assay has been shown to give reliable results for minute amounts of endotoxin in water (4), measurements in blood still present difficulties. Firstly, there is still disagreement about the best way of preparing standard curves (5–8) and secondly, an optimal method for eliminating plasma inhibitors in the endotoxin assay has not yet been established. Among various pretreatment procedures aimed at avoiding inhibitory activities in plasma, dilution and heating (9, 10) and perchloric acid treatment (11) have been widely used. Studies comparing these methods (11, 12) have produced conflicting results.

The aim of this study was to define the optimal way of obtaining standard curves for a chromogenic assay and to re-evaluate plasma pretreatment procedures.

Materials and Methods

1. Subjects

Venous blood was collected from 12 healthy individuals (age range 25 to 55 years) and 24 chronic alcoholics (7 with fatty liver and 17 with liver cirrhosis, age range 60 to 65 years). The diagnosis was based on liver biopsy in all cases.

2. Blood sampling

After cleaning the skin twice with aqueous ethanol, volume fraction 0.6 and with Kodan-spray (Schülke & Mayr GmbH, Norderstedt, F. R. G.), 3 ml blood samples were collected from the antecubital vein into pyrogen-free syringes containing 75 units of NH_4-heparin as previously described (13). Care was taken to avoid air bubbles in the syringes. Platelet-rich plasma was prepared by centrifugation at 150 *g* for 20 min at 0 °C. The separated plasma was stored at −80 °C in pyrogen-free polyethylene tubes (Eppendorf, Hamburg, F. R. G.).

3. Chromogenic assay

Hydrolysis of the chromogenic substrate was determined in two different ways: firstly as described in the original method used (6) by incubation for three minutes and stopping the reaction with acetic acid (endpoint determination), and secondly by checking the linearity of the indicator reaction used in the test by measuring hydrolysis kinetics of the chromogenic substrate (continuous assay).

3.1 Endpoint determination

Endotoxin was measured in plasma by a modification of the chromogenic substrate test (Kabi-Vitrum, Stockholm, Sweden) as described by *Friberger* et al. (6). The plasma was diluted 1:10 with ice-cold pyrogen-free water. In order to prepare a large number of samples for parallel incubation, the diluted plasma samples were allowed to stand for a maximum of 30 minutes on ice. Storage of these plasma samples, diluted 1:10 on ice, resulted in no loss of endogenous or added endotoxin over a period of up to two hours. The diluted plasma samples were then heated for 5 min at 75 °C. Fifty µl of *Limulus* amoebocyte lysate was added to 50 µl of the heated plasma. After a preincubation time of 25 min (37 °C), 100 µl of substrate (S-2423, Ac-Ile-Glu-Gly-Arg-*p*-nitroanilide, 1.4 mmol/l) dissolved in Tris buffer (25 mmol/l, pH 9.0) were added and incubated at 37 °C for exactly 3 min. The reaction was stopped by adding 100 µl 3.3 mol/l acetic acid. Absorbance was measured at 405 nm. The test was performed in sterile polyethylene tubes. All procedures were performed in a laminar flow bank (Captair 2004, Hermann Waldner KG, Wangen im Allgäu, F. R. G.). In accordance with the instructions provided by the manufacturer all determinations were performed in duplicate.

Two blank values were run in parallel with each test: one in which the same volume of pyrogen-free water was added instead of 50 µl diluted plasma, and a second with which the intrinsic absorbance of the plasma was to be measured, and in which *Limulus* amoebocyte lysate was replaced by 50 µl pyrogen-free water. Otherwise, the two blank values were treated in the same way as the plasma measurement values.

3.2 Continuous measurement

The kinetics of the enzymatic hydrolysis of the chromogenic substrate were measured at 37 °C for 0–10 min, using sterile microtitre plates in a micro-processor controlled reader (EAR400AT, SLT Lab. Inst., Salzburg, Austria), which was placed in a laminar flow bank. Preparation of the plasma samples (including preincubation and addition of substrate) was exactly as described under 'endpoint determination', with the sole exception that the volumes were reduced to 40 µl sample, 40 µl *Limulus* amoebocyte lysate and 80 µl substrate buffer. Measurement of the change in the absorbance was carried out at intervals of 30 seconds at 405 nm.

4. Standard curves and calculation of endogenous endotoxin

Endotoxin standards were prepared from *E. coli* O 111:B 4 (Kabi Vitrum, Stockholm, Sweden; 1 ng = 12 endotoxin units (EU). According to the information of the manufacturer the endotoxin unit (EU) is determined against the EC5 standard of the Food and Drug Administration (FDA) or lot F, USP reference standard endotoxin and controlled under FDA licence No 709. To each 100 µl 1:5 diluted plasma sample, 100 µl of pyrogen-free water or 100 µl of different standard endotoxin solutions (12.5–100 ng/l) were added before heating. This gave final endotoxin concentrations of 0, 6.25, 12.5, 25, 37.5 and 50 ng/l. After adding the respective amount of standard endotoxin solution, the samples were ice-cooled for a maximum of five minutes before heating.

To plot the standard curves, the measured changes in absorbance were used for each standard value after subtracting the value for the plasma sample (with no addition of exogenous endotoxin standard).

The endogenous endotoxin concentration was calculated as follows:

$$\text{Endotoxin } (10^3 \text{ EU/l}) = [A_{pl} - A_{H_2O} - A_{pl\ \text{blank}}] \times \frac{0.2 \text{ EU/ml} - 0.1 \text{ EU/ml}}{\Delta A_{0.2\,EU} - \Delta A_{0.1\,EU}} \times 10$$

where
A_{pl} = absorbance for plasma
A_{H_2O} = absorbance for water (both after incubation with *Limulus* amoebocyte lysate)
A_{pl} blank = absorbance for plasma blank
$\Delta A_{0.2\,EU}$ and $\Delta A_{0.1\,EU}$ were read off the standard curve.
Fraction bar = reciprocal value of the slope of the standard curve.

5. Recovery

In order to establish how much exogenous endotoxin is lost by heating, another endotoxin standard with a final concentration of 25 ng endotoxin per litre 1:10 diluted plasma was prepared by adding endotoxin to each plasma after heating. The increase in absorbance in the samples with added endotoxin after heating was taken as 100%, and the recovery of the endotoxin added to the sample before heating was calculated as the recovered percentage as follows:

$$\text{Recovery } \% = A/B \times 100,$$

where
A = net increase in absorbance in the sample with endotoxin added before treatment,
B = net increase in absorbance in the sample with endotoxin added after treatment. In each run of the assay, a standard curve in pyrogen-free water was also prepared as a control.

6. Acid treatment

In order to compare the results obtained with pretreatment of the plasma by heating as described in section 3.1 with those obtained with the method proposed by *Obayashi* (11) in which plasma is treated with acid before endotoxin determination, two variations of the test with acid pretreatment were performed as follows.

6.1 Plasma samples from 22 subjects (8 healthy individuals, 7 alcoholics with fatty liver and 7 alcoholics with liver cirrhosis) were treated using the original method of *Obayashi* (11). Plasma (180 µl) was mixed with either 20 µl pyrogen-free water or endotoxin standard solution at a concentration of 0–50 ng exogenous endotoxin per litre of diluted (3:20) plasma.

6.2 It was considered that recovery might be better if the plasma was diluted further. Plasma samples (100 µl) from the 22 subjects mentioned in 6.1 were mixed with 100 µl water or endotoxin standard solution at an exogenous endotoxin concentration of 0–50 ng per litre of diluted (1 : 12) plasma.

The diluted plasma samples (200 µl) pretreated as described under 6.1 and 6.2 were mixed with 400 µl of 0.32 mol/l $HClO_4$, incubated for 20 min at 37 °C and centrifuged at 1000 *g* for 15 min at 0 °C. An aliquot (100 µl) of each supernatant was neutralized by adding of 100 µl 0.18 mol/l NaOH; tests on 6 plasma samples showed that this treatment resulted in a pH of 7–7.5. Final plasma dilutions were therefore 3 : 20 and 1 : 12 respectively. Another endotoxin standard with a final concentration of 25 ng endotoxin per litre plasma (diluted 3 : 20 or 1 : 12) was prepared by adding endotoxin to the neutralized supernatant. The recovery rate of added endotoxin was calculated as described in section 4. For each run of the assay, endotoxin standard (25 ng/l) was prepared in pyrogen-free water both with and without $HClO_4$ pretreatment.

7. Self-prepared control samples

To evaluate the variation in endotoxin concentration over time from one determination to the next, plasma from a healthy individual which was both spiked and non-spiked with exogenous endotoxin (25 ng/l) was divided into 0.5 ml portions in sterile polyethylene tubes and stored at −80 °C. The above sample was measured weekly for 1 month and thereafter in parallel with every endotoxin determination of the study over a period of 8 months to obtain a measure of the precision of the assay and the stability of endotoxin during storage.

8. Statistical analysis

For statistical evaluation, *Wilcoxon-Mann-Whitney*'s U-test was used. The results were expressed as means ± SD.

Results

1. Standard curves

The kinetics of the hydrolysis of the chromogenic substrate with endotoxin standards in water and in plasma from healthy individuals (fig. 1) was linear for 3 min. The endogenous endotoxin concentrations in the plasma from the healthy individuals never exceeded 60 endotoxin units per litre (= 5 ng/l). The net increase in the absorbance (ΔA) obtained in the plasma samples was 4 to 5 times greater than that observed in water. When endotoxin standards were added to plasma from two patients with very high endogenous endotoxin concentrations (> 2400 endotoxin units per litre = > 200 ng/l), deviations from linearity were seen after 2 and 2.5 min, respectively.

The slope of the standard curve in each plasma exhibited marked interindividual variation, particularly in the alcoholics (fig. 2). Each standard curve was linear up to 37.5 ng endotoxin per litre of diluted (1 : 10) plasma (corresponding to a final concentration of 375 ng/l = 4500 endotoxin units per litre of plasma; fig. 2).

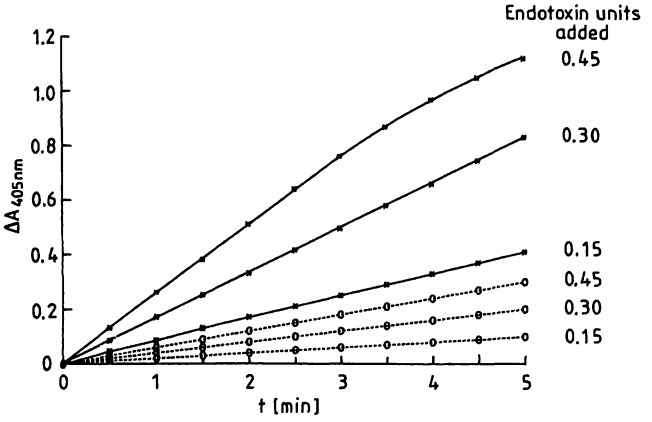

Fig. 1. Kinetics of the hydrolysis of the chromogenic substrate by endotoxin standards in distilled pyrogen-free water (o– –o) and in plasma (x–x) from a healthy subject, using microtitre plates. The net increase in absorbance ($\Delta A_{405\,mm}$) was obtained by subtracting the value of water or plasma without endotoxin. The assay was performed in 40 µl samples.

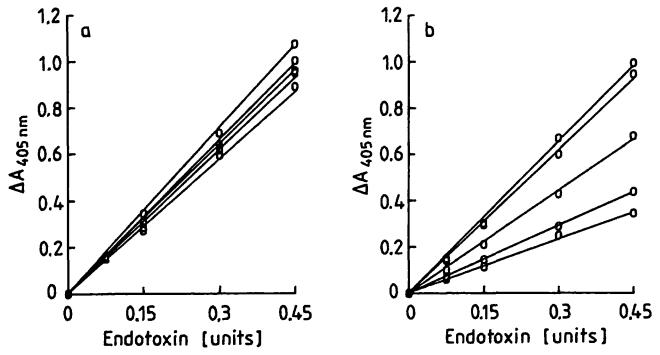

Fig. 2. Standard curves of endotoxin in plasma samples of 5 healthy subjects (a) and 5 patients with alcoholic liver disease (b). Determinations were performed using the endpoint determination (sample volume: 50 µl). Note the marked variation of the slope of the standard curves in the group with alcoholic liver disease.

Linearity was also observed up to 3 min for samples with endotoxin concentrations up to 50 ng per litre diluted (1 : 10) plasma when the hydrolysis of the chromogenic substrate was measured continuously (fig. 1). For the two plasma samples containing very high endogenous endotoxin concentrations, the linear parts of the kinetic curves were extrapolated to 3 min and used to draw standard curves.

2. Recovery of endotoxin by the heating method

The recovery of added endotoxin was 91.3 ± 13.3% (mean ± SD) in plasma from controls (fig. 3), and 89.8 ± 15.1% in plasma from the total group of alcoholics. The recovery rate in plasma from cirrhotics (87.4 ± 17.3%) was not significantly different from that in plasma from patients with fatty liver (95.7 ± 4.4%).

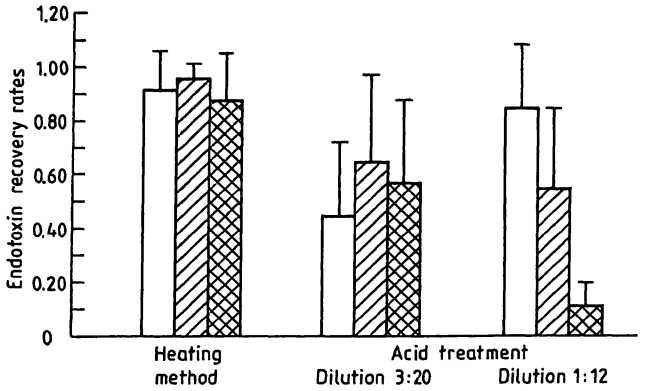

Fig. 3. Comparison of the three different procedures for eliminating factors interfering with the endotoxin determination using a chromogenic substrate. The recovery rates of endotoxin standard with these procedures were compared in plasma samples obtained by the heating method from 12 healthy subjects (normal), 7 patients with alcoholic fatty liver and 17 patients with alcoholic cirrhosis.
For both methods using perchloric acid, the number of subjects in the three troups was 8, 7 and 7, respectively.
□ normal ▨ fatty liver ▩ cirrhosis

3. Recovery of endotoxin by the $HClO_4$ method

The recovery of added endotoxin was highly variable, both in plasma from controls (44.3 ± 27.0%) and in plasma from alcoholics with cirrhosis (58.5 ± 29.5%) when the acid method 1 (final plasma dilution 3:20) was used. There was no significant difference in the recovery rates between healthy subjects and the two groups with alcoholic liver disease (fig. 3). The recovery rates of endotoxin in plasma both from controls and alcoholics with this method were significantly lower ($P < 0.005$, $P < 0.01$) than those obtained with the heating method.

The recovery of added endotoxin from plasma samples of controls was 84.3 ± 22.9% when acid method 2 (final plasma dilution 1:12) was used. Using this method, the recovery of endotoxin from plasma of alcoholics with fatty liver varied greatly (57.9 ± 27.6%) and was lower than in healthy subjects ($P < 0.05$). The lowest recovery rate was noted in cirrhotics (11.6 ± 7.5%). The recovery rates of endotoxin in plasma from patients with fatty liver and from cirrhotics by this method were significantly lower than those obtained with the heating method ($P < 0.025$, $P < 0.005$) (fig. 3).

4. The endotoxin standard in water

Endotoxin standards (25 ng/l) in pyrogen-free water, always gave lower absorbance values than those in the plasma samples with addition of endotoxin added before heating (fig. 1).

5. Self-prepared control samples

As shown in figure 4, the values of the self-prepared control samples remained fairly constant for a period of almost 8 months. The mean value ± SD of all determinations of the self-prepared control samples was 300 ± 45 endotoxin units per litre (n = 22). The results obtained by the endpoint method ($\bar{x}$ ± SD = 290 ± 26 endotoxin units per litre; n = 10) were comparable to those obtained by the continuous measurement using microtitre plates ($\bar{x}$ ± SD = 312 ± 56 endotoxin units per litre; n = 12). The coefficient of variation between days of determination was 15% for all values (n = 22). Related to the periods when only the "endpoint" or the "continuous" method was used, the variation coefficient was 8.9% (n = 10) and 18.1% (n = 12) respectively.

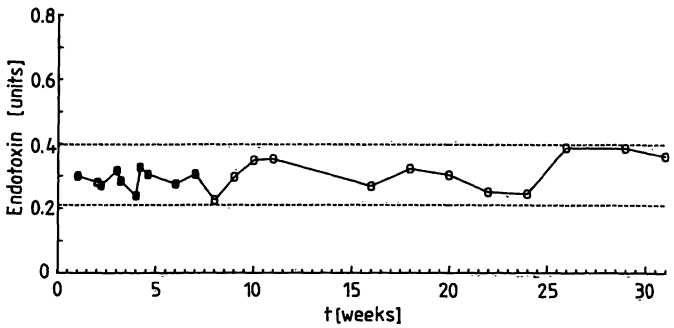

Fig. 4. Repeated determinations of endotoxin in the self-prepared control samples. The first 10 determinations were performed using the endpoint determination (solid circles), the following 12 determinations using the continuous assay (open circles). The dotted line represents the means ± 2 SD of all determinations.

Discussion

After a sensitive chromogenic substrate assay had been developed for the determination of endotoxin concentration in blood, several authors (5, 7, 10, 12, 14) tried to establish the optimal assay conditions. A number of important points, such as heparin concentration in plasma, inactivation temperature for inhibitors, assay pH, influence of Ca^{2+}, Mg^{2+} or Na^{+}, stability of lysate and endotoxin standard, recovery of added endotoxin from platelet-rich and platelet-poor plasma have already been exhaustively investigated (5, 7, 10, 12, 15). However, the questions of how to establish the standard curve for calculating plasma endotoxin concentration and how to pretreat the plasma sample adequately to minimize binding or inactivation of endotoxin have not yet been satisfactorily resolved (8, 15–19). The differences in the methods used to pretreat plasma and establish standard curves might influence the results of plasma endotoxin determinations in patients with various diseases.

In previous studies on the determination of endotoxin in the plasma of patients with various diseases, standard curves of endotoxin were prepared either in pyrogen-free water (11, 15, 19) or in pooled plasma from healthy subjects (7). Our own results show that the kinetics of the hydrolysis of the chromogenic substrate in plasma differs from that in pyrogen-free water. The net increase in absorbance of the plasma was 4 to 5 times greater than that of water. *Harris* et al. (12) also reported higher absorbance readings with dilutions of endotoxin in plasma compared with similar dilutions in water, and supposed that a physicochemical change of endotoxin in plasma might lead to enhanced reactivity. On the basis of these results, we suggest that standard curves in water should not be used for the calculation of endotoxin concentration in plasma. The present study further reveals that the standard curve in each plasma sample exhibits marked interindividual variation. Since the slope of the standard curve varies greatly, endotoxin plasma concentration in each patient must be calculated from its own individual standard curve. Calculations performed using a common standard curve prepared for pooled plasma will frequently lead to false results. *Urbaschek* et al. (20) also emphasized this point on the basis of their results in a densitometric kinetic *Limulus* lysate microtitre test.

In the present study, we have been able to show that the mean recovery rate of exogenous endotoxin with the heating method was about 90% and generally satisfactory both in control plasma and in plasma from patients. But the fact that the recovery of exogenous endotoxin added to the plasma before heating was usually less than 100% supports the assumption that the spiking of plasma should be done in samples both before and after heating. This procedure permits the calculation of the amount of endogenous endotoxin lost by heating, and a more accurate calculation of the endotoxin concentration in a given plasma. The problem mentioned above, namely that the percentage of endotoxin, bound or inactivated at the time of blood sampling is not measured (8, 15–19), is, however, not resolved in this way.

An endotoxin assay with an individual standard curve for each plasma certainly improves reliability, but makes the endotoxin determination more expensive. It is therefore reasonable to attempt to reduce sample and lysate volume to minimize costs. Using the endpoint assay in plastic tubes, we obtained satisfactory results with 50 µl of samples and lysates. When microtitre plates were used for the continuous assay a further volume reduction to 40 µl was achieved. A similar reduction in the volume of plasma and lysate has been obtained by others (14).

In the present study, the recovery rates of endotoxin from spiked samples were lower with the $HClO_4$ method than with the heating method. In contrast to our study, other authors (11, 19) have reported a recovery of endotoxin with the $HClO_4$ method of almost 100%.

The reason for the discrepancy between this figure and our own results is difficult to explain. One factor which might contribute to the high endotoxin recovery by *Obayashi* (11) and *Yajima* et al. (19) is the fact that these authors divided the net increase of absorbance in plasma by that obtained in water to calculate their recovery rates. We have demonstrated that, for a given amount of exogenous endotoxin, the increase in absorbance is higher in neutralized supernatant from plasma treated with $HClO_4$ than in the same volume of water. With their mode of calculation *Obayashi* (11) and *Yajima* et al. (19) might have overestimated the percentage recovery rate. It has been generally accepted that endotoxin activities of lipopolysaccharides, including *Limulus* amoebocyte lysate gelating activity, are located in their lipid A moiety (21, 22). Acid hydrolysis has been widely used to isolate lipid A from various lipopolysaccharides (23–25). Therefore, $HClO_4$ treatment might split at least a part of endogenous and exogenous endotoxin, leading to the formation of free lipid A, which would then be found in the precipitate after centrifugation. In addition endotoxin bound to protein might also precipitate. This precipitated part of endotoxin activity is likely to be missed by the $HClO_4$ method.

The observed difference in recovery rates for the diluted (3 : 20) plasma as compared with the diluted (1 : 12) plasma, using the $HClO_4$ method, supports the assumption that recovery of endotoxin in the supernatant depends on the amount of protein or other compounds that can bind or inactivate endotoxin in the acidified mixtures.

The results of the determination in the self-prepared control samples further support the assumption that the method described for endotoxin determination in plasma has an acceptable level of reproducibility. In addition, they show that under the conditions of our study the endotoxin concentration in plasma remains nearly unchanged for a period of eight months.

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References

1. Nolan, J. P. (1981) Endotoxin, reticuloendothelial function, and liver injury. Hepatology *1*, 458–465.
2. Sturk, A. & ten Cate, J. W. (1985) Endotoxin testing revisited. J. Clin. Microbiol. *4*, 382–385.
3. Levin, J. (1982) The Limulus test and bacterial endotoxins: some perspectives. In: *Endotoxins and their detection with the Limulus amebocyte lysate test* (Watson, S., Levin, J. & Novitsky, T. J., eds.) pp. 7–24 New York: A. R. Liss.
4. Iwanaga, S., Morita, T. & Harada, T. (1978) Chromatogenic substrates for horseshoe crab clotting enzyme: it's application for the assay of bacterial endotoxins. Hemostasis *7*, 183–188.
5. Sturk, A., Joop, K., ten Cate, J. W. & Thomas, L. L. M. (1985) Optimalization of a chromogenic assay for endotoxin in blood. In: *Bacterial Endotoxins: structure, biomedical significance and detection with Limulus amebocyte lysate test* (ten Cate, J. W., Büller, H. R., Sturk, A. & Levin, J., eds.) pp. 117–136 New York: A. R. Liss.
6. Friberger, P. (1985) The design of a reliable endotoxin test. In: *Bacterial endotoxins: structure, biomedical significance and detection with Limulus amebocyte lysate test* (ten Cate, J. W., Büller, H. R., Stark, A. & Levin, J., eds.) pp. 139–149 New York: A. R. Liss.
7. Sturk, A., Janssen, M. E., Muylaert, F. R., Joop, K., Thomas, L. L. M. & ten Cate, J. W. (1987) Endotoxin testing in blood. Progr. Clin. Biol. Res. *231*, 371–385.
8. Piotrowics, B. I., Edlin, S. E. & McCartney, A. Ch. (1985) A sensitive chromogenic Limulus amoebocyte lysate microassay for detection of endotoxin in human plasma and in water. Zbl. Bakt. Hyg. *A260*, 108–112.
9. Cooperstock, M. S., Tucker, R. P. & Baublis, J. V. (1975) Possible pathogenic role of endotoxin in Reyes syndrome. Lancet *I*, 1272–1274.
10. Friberger, P., Knös, M. & Mellstam, L. (1982) A quantitative endotoxin assay utilizing LAL and a chromogenic substrate. In: *Endotoxin and their detection with the Limulus amebocyte lysate test* (Watson, S., Levin, J. & Novitsky, T. J., eds.) pp. 195–206 New York: A. R. Liss.
11. Obayashi, T. (1984) Addition of perchloric acid to blood samples for colorimetric limulus test using chromogenic substrate: comparison with conventional procedures and clinical application. J. Lab. Clin. Med. *104*, 321–330.
12. Harris, R. I., Stone, P. C. W. & Stuart, J. (1983) An improved chromogenic substrate endotoxin assay for clinical use. J. Clin. Pathol. *36*, 1145–1149.
13. Bode, C., Kugler, V. & Bode, J. C. (1987) Endotoxemia in patients with alcoholic and non-alcoholic cirrhosis and in subjects with no evidence of chronic liver disease following acute alcohol excess. J. Hepatology *4*, 8–14.
14. Scully, M. F., Newman, Y. M., Clark, S. E. & Kakkar, V. V. (1980) Evalution of a chromogenic method for endotoxin measurement. Thromb. Res. *20*, 263–270.
15. Tachiyama, G., Sakon, M., Kambayashi, J., Ohshiro, T. & Mori, T. (1986) Chromogenic assay of endotoxin in platelet poor or rich plasma. Thromb. Res. *37*, 309–317.
16. Pearson, F. C., Dubczak, J., Weary, M., Bruszer, G. & Donohue, G. (1985) Detection of endotoxin in plasma of patients with gram-negative bacterial sepsis by the Limulus amoebocyte lysate assay. J. Clin. Microbiol. *21*, 865–868.
17. Bigatello, L. M., Broitman, S. A., Fattori, L., Di Paoli, M., Pontello, M., Bevilacqua, G. & Nespoli, A. (1987) Endotoxemia, encephalopathy, and mortality in cirrhotic patients. Am. J. Gastroenterol *82* 11–15.
18. Büller, H. R., ten Cate, J. W., Sturk, A. & Thomas, L. L. (1985) Validity of the endotoxin assay in post surgical patients. In: *Bacterial endotoxins: structure, biomedical significance and detection with Limulus amoebocyte lysate test* (ten Cate, J. W., Büller, H. R., Sturk, A. & Levin, J., eds.) pp. 405–416 New York: A. R. Liss.
19. Yajima, Y., Fukuda, I., Otsuki, M., Suzuki, H., Ota, S., Ishii, M., Mori, K. & Goto, Y. (1985) Endotoxemia in liver diseases: detection by a quantitative assay using chromogenic substrate with perchloric acid pretreatment. Tohoku J. Exp. Med. *147*, 411–419.
20. Urbaschek, B., Becker, K. P., Ditter, B. & Urbaschek, R. (1985) Quantification of endotoxin and sample-related interferences in human plasma and cerebrospinal fluid using a kinetic Limulus amoebocyte lysate microtiter test. In: *Microbiology* (Leive, L., ed.) pp. 39–43 Washington DC, American Society for Microbiology.
21. Galanos, C., Lüderitz, O., Rietschel, E. Th. & Westphal, O. (1977) Newer aspects of the chemistry and biology of bacterial lipopolysaccharides with special reference to their lipid A component. In: *International Review of Biochemistry* (Goodwin, T., ed.) Vol. 14: Biochemistry of Lipids II. pp. 239–335 Baltimore, University Park Press.
22. Rietschel, E. Th., Galanos, C., Lüderitz, O. & Westphal, O. (1982) Chemical structure, physiological function and biological activity of lipopolysaccharides and lipid A. In: *Immunopharmacology and regulation of leukocyte function* (Webb, D. R., ed.) pp. 183–229 New York, Marcel Dekker.
23. Galanos, C., Lüderitz, O. & Westphal, O. (1971) Preparation and properties of antisera against the lipid A component of bacterial lipopolysaccharides. Eur. J. Biochem. *24*, 116–122.
24. Galanos, C. & Lüderitz, O. (1975) Electrodialysis of lipopolysaccharides and their conversion to uniform salt forms. Eur. J. Biochem. *54*, 603–610.
25. Wollenweber, H. W., Seydel, U., Lindner, B., Lüderitz, O. & Rietschel, E. Th. (1984) Nature and location of amide-bound (R)-3-acyloxyacyl groups in lipid A of lipopolysaccharides from various gram-negative bacteria. Eur. J. Biochem. *145*, 256–272.

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